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Localization of the ActA Polypeptide of *Listeria monocytogenes* in Infected Tissue Culture Cell Lines: ActA Is Not Associated with Actin "Comets"

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The ActA protein of the gram-positive pathogen *Listeria monocytogenes* is a 90-kDa polypeptide required for interaction of the bacteria with components of the host cell microfilament system to generate intra- and intercellular movement. To study the localization, distribution, and expression of the ActA polypeptide in *L. monocytogenes* grown either in broth culture or in infected tissue culture cells, we first isolated ActA by monoclonal antibody-based immunoaffinity chromatography. Polyclonal rabbit antisera raised against purified ActA revealed that ActA was associated with the cell wall and exposed on the surface of the bacteria, readily accessible to ActA antibodies. In contrast, a C-terminally truncated ActA1 polypeptide expressed by the isogenic *actA1* mutant was detected only in the supernatant fluids. Immunofluorescence microscopy and electron microscopic studies using immunogold labeling showed that ActA was present on the surface of the bacteria infecting PtK_2 and J774 cells at all stages of the infection cycle and was not found to be associated with the actin "tail" of individual bacteria. For the isogenic *actA1* mutant strain, which grew as microcolonies within infected cells, only diffuse staining of the secreted ActA1 polypeptide in the host cytoplasm was observed. The ActA polypeptide therefore appears to be required in the initiation of actin accumulation by the bacterium and is apparently not directly involved in the generation of the actin tail. Analysis of strains of several *L. monocytogenes* serotypes indicated microheterogeneity in the molecular weights of the ActA polypeptides of individual strains and led to the detection of a serotype 3a strain that does not produce ActA.

Listeria monocytogenes is a widespread, rapidly growing, gram-positive bacterium that causes food-borne infections in animals and humans, with severe implications, especially for newborns and immunocompromised individuals (5). As a facultative intracellular pathogen, *L. monocytogenes* is capable of surviving and multiplying in a variety of mammalian cells (4, 9, 12). Even though intracellular growth is a crucial step in the course of infection, several other critical steps are required in the manifestation of the infection cycle. These include invasion, escape from the phagolysosomal compartment (8, 15), and intercellular spread (see reference 14 and references therein). Once within the cytoplasm of the host cell, the otherwise nonmotile bacteria accumulate actin filaments, which subsequently form tail-like structures that enable rapid bacterial movement. Some bacteria are extruded from the infected cell in macrovilli or pseudopod-like structures, which can be internalized by neighboring cells. At this point the bacteria lyse the double-membrane vacuole and initiate another cycle of growth and infection (4, 18, 19).

Recently, we and others have identified an *L. monocytogenes* bacterial surface protein that is involved in actin accumulation within the infected host cell (3, 7). This protein, termed ActA, has an apparent molecular mass of 90 kDa. Bacterial mutants with mutations in the *actA* gene cannot accumulate actin and are therefore incapable of intracellular and cell-to-cell spread. When tested in a mouse infection model, such *actA* mutants are highly attenuated for virulence, demonstrating that the actin-dependent phenomenon of bacterial movement within and between host cells is a key virulence mechanism of *L. monocytogenes*. However, apart from the involvement of actin and ActA, the basis of this highly elaborate mechanism of bacterial motility and spreading is not understood. It is, for example, not clear whether actin directly interacts with ActA, nor is it known how polarized bacterial movement is established within the cytoplasm. Also, no information is available on the localization of the ActA polypeptide on the bacteria within the infected cell, particularly with respect to the actin "tails," which are the basis of intracellular motility of these bacteria (4, 17, 18).

In this communication we describe the generation of a monoclonal antibody (MAb) that was used to purify ActA in a single step by immunoaffinity chromatography. By using a specific polyclonal antiserum raised against purified ActA, the expression and location of the ActA polypeptide in bacterial cells grown either in vitro or within the infected eucaryotic host cell were determined. Finally, immunoblotting experiments with this antiserum allowed detection of the different ActA polypeptides in *L. monocytogenes* strains of several serotypes and revealed a naturally occurring variant that did not express the ActA polypeptide.

MATERIALS AND METHODS

Bacterial strains, media, and reagents. The weakly hemolytic *L. monocytogenes* strain EGD (serotype 1/2a) and its isogenic *actA1* and *prfA1* mutants have been described previously (2, 3). Strains of other serotypes of *L. monocytogenes* are listed in Table 1 and have been described previously (20). *Listeria* strains were grown in brain heart infusion broth (Difco) at 37°C, with 5 μg of erythromycin per

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TABLE 1. Detection of ActA in different serotypes of *L. monocytogenes*

Serotype	*L. monocytogenes* strain	Presence of ActA[a]
1/2 a	EGD	+
	SLCC 2371	+
	NCTC 7973	+
1/2 b	SLCC 2755	+
1/2 c	SLCC 2372	+
3 a	SLCC 2373	−
3 b	SLCC 2540	+
	SLCC 5311	+
	SLCC 5543	+
3 c	SLCC 2479	+
4 a	SLCC 2374	+
	L 99	+
4 ab	NCTC 10528	−
4 b	SLCC 1444	+
	SLCC 3551	+
	SLCC 4013	+
	SLCC 5489	+
4 c	SLCC 2376	+
	SLCC 4925	+
4 d	SLCC 2377	+
4 e	SLCC 2378	+
7	SLCC 2482	+

[a] As detected by immunoblotting.

ml in the cases of the *actA1* and *prfA1* mutants. All chemical reagents were purchased from Sigma (Deisenhofen, Germany) unless indicated otherwise.

SDS-PAGE, immunoblotting, and sequence analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (11) with 7.5 and 10% polyacrylamide gels. For the determination of apparent molecular weights, the low-molecular-weight marker from Bio-Rad was used. For staining of the gels either Coomassie brilliant blue R-250 or a silver staining kit (Bio-Rad) was used. Immunoblotting was performed with a semidry device (10), using Immobilon P membranes (Millipore). For screening of hybridoma supernatants, samples of concentrated *L. monocytogenes* supernatants were loaded onto slotless gels. After blotting of the gels, several hybridoma clones could be tested simultaneously with a "miniblotting" apparatus (Biometra, Göttingen, Germany). The blots were reacted with horseradish peroxidase-conjugated secondary antibody, using chloronaphthol and hydrogen peroxide in phosphate-buffered saline (PBS) as the substrate. When necessary, a sensitive chemiluminescence-based immunoblot assay (ECL; Amersham Buchler, Braunschweig, Germany) was used according to the instructions of the vendor.

For sequence analysis, separated polypeptides were blotted onto polyvinylidene difluoride membranes (Problot; Applied Biosystems) and analyzed with an Applied Biosystems gas phase sequenator (model A470) equipped with an on-line phenylthiohydantoin (PTH) amino acid analyzer.

***Listeria* SDS cell wall extracts.** Exponentially growing cultures were harvested by centrifugation, and bacterial pellets were immediately resuspended in boiling SDS-sample buffer and heated for a further 5 min. Following centrifugation, the supernatants were aliquoted and kept at −70°C. Alternatively, pellets were rinsed twice with PBS and incubated with 1% (wt/vol) SDS in PBS for 15 min at 37°C (7). When necessary, extracts were concentrated by using Centriprep-10 concentrators (Amicon, Beverly, Mass.).

Antigen preparation. (i) Preparation of concentrated bacterial supernatants. In order to obtain defined bacterial culture supernatants, the brain heart infusion medium was prefiltered, using a cutoff of 10 kDa (Minitan; Millipore), before it was inoculated with bacteria. Exponentially growing cultures (optical density at 600 nm = 0.6) were harvested by centrifugation. Phenylmethylsulfonyl fluoride (0.2 mM) and NaN_3 (0.05%, wt/vol) were added to the supernatants, which were then immediately concentrated up to 200-fold by ultrafiltration at 4°C, retaining substances of greater than 10 kDa. The concentrated supernatants were stored in aliquots at −70°C.

(ii) Ammonium sulfate precipitation. Concentrated *L. monocytogenes* EGD supernatants were sequentially precipitated with ammonium sulfate at 4°C (from 10 to 80% final saturation). Pelleted fractions were dialyzed extensively against PBS and analyzed by SDS-PAGE and immunoblotting.

(iii) Preparative SDS-PAGE. The ammonium sulfate preparations were separated by preparative SDS-PAGE using 10% polyacrylamide gels (11). The gels were stained with Coomassie brilliant blue, and relevant bands were excised. Electroelution was performed in Laemmli buffer directly into dialysis tubes, which were then dialyzed extensively against 0.1 M $(NH_4)_2CO_3$. The dialyzed eluates were lyophilized, dissolved in PBS, analyzed by SDS-PAGE, and stored in aliquots at −70°C.

Antibody production. (i) MAbs. Female BALB/c mice were immunized at 3-week intervals intraperitoneally with the 90-kDa ActA polypeptide, which had been electroeluted from preparative gels of the ammonium sulfate-enriched fraction, using 10 to 20 μg of protein per injection with Freund's complete adjuvant for the first immunization and incomplete adjuvant for two subsequent booster injections. Antisera obtained were assayed by enzyme-linked immunosorbent assay (ELISA) and immunoblotting. The spleen cells from the mouse exhibiting the strongest reaction were fused with myeloma cells (X63Ag8) by standard protocols. Myeloma and hybridoma cells were maintained in OPTI-MEM (GIBCO) supplemented with 5% fetal calf serum and standard concentrations of penicillin-streptomycin and glutamine. Hybridoma selection was performed with azaserine-hypoxanthine at a concentration recommended by the manufacturer. Positive colonies were subcloned twice by limiting dilution. Immunoglobulin subclasses of MAbs were determined by using an isotyping kit (Bionetics). Supernatants were screened for antibody production by ELISA and immunoblotting. MAbs of the immunoglobulin G subclass were purified from hybridoma supernatants by using protein A-Sepharose (see below).

(ii) Polyclonal antibodies. Two rabbits each were immunized with affinity-purified ActA polypeptides (100 μg per injection) thrice at 3-week intervals by standard immunization procedures. The production of antisera against synthetic

peptides to the corresponding amino acid sequences of the ActA polypeptide has been described previously (3).

ELISA. For ELISA, 96-well microtiter plates (Maxisorp; Nunc) were coated with *L. monocytogenes* concentrated supernatant, diluted 1:100 in 0.1 M Na-carbonate buffer (pH 9.6), for 12 to 14 h at 4°C. Alternatively, ammonium sulfate-purified protein fractions were used for coating. Following saturation with 10% (vol/vol) fetal calf serum in PBS, the plates were washed three times with PBS, after which diluted antisera or hybridoma culture supernatants were added and incubated for at least 1 h. Bound antibodies were detected by incubation with horseradish peroxidase-conjugated goat anti-mouse antibodies (Dianova, Hamburg, Germany) for 1 h followed by extensive washing. *o*-Phenylenediamine and hydrogen peroxide were used as substrates.

Affinity purification of ActA. The ActA-specific MAbs N20, N81, and N111 were purified from hybridoma culture supernatants with a protein A-Sepharose column. Supernatants were adjusted to 3 M NaCl–0.1 M sodium borate (pH 8.9). MAbs were eluted with 0.1 M citrate (pH 3.0) and immediately neutralized with 0.2 M Tris-Cl (pH 8.8). The MAb-containing fractions were dialyzed against 0.1 M sodium borate (pH 8.4) containing 0.5 M NaCl. Purified MAbs were coupled to CNBr-activated Sepharose 4B (Pharmacia), according to standard protocols, using 10 mg of antibody per g of Sepharose. Unless stated otherwise, all experiments were performed at room temperature. Immobilized MAbs were incubated overnight at 4°C on a rotating device (batch method) with concentrated *L. monocytogenes* supernatants containing 50 mM phosphate buffer (pH 6.8), 10% (vol/vol) glycerol, 0.5 mM EDTA, 1 mM β-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride. For preparative purposes, the affinity matrix was filled into an appropriate column and washed with 10 volumes of 50 mM phosphate buffer (pH 6.8) containing 10% (vol/vol) glycerol, 0.5 mM EDTA, and 1 mM β-mercaptoethanol. To remove nonspecifically bound material, the Sepharose was washed with 3 volumes of the same buffer containing 1.5 M NaCl. Before the elution was begun, the Sepharose was washed with 5 volumes of phosphate buffer-glycerol. Finally, the bound antigen was eluted from the column with 3.7 M $MgCl_2$ in 50 mM sodium phosphate buffer (pH 6.8) containing 10% (vol/vol) glycerol. Aliquots of eluted fractions were precipitated with 10% (wt/vol) trichloroacetic acid and analyzed by SDS-PAGE. Relevant fractions were pooled, desalted, and concentrated by repeated centrifugation in Centriprep-10 concentrators (Amicon) at 4°C with PBS. Protein concentrations were determined according to the method of Bradford (1), using bovine serum albumin as the standard.

Affinity purification of polyclonal ActA antibodies. Affinity-purified ActA (2 mg) was coupled to 1 g of CNBr-activated Sepharose 4B by standard protocols. For the antibody purification, 10 ml of the ActA antiserum was passed through a column that contained the affinity matrix. Unspecifically bound material and low-affinity antibodies were removed with 0.1 M acetate buffer (pH 4.8) containing 0.5 M NaCl. Antibodies were then eluted with 0.2 M acetate buffer (pH 2.7) containing 0.5 M NaCl and immediately neutralized with 0.2 M Tris-Cl (pH 8.8).

Immunofluorescence labeling of listeriae grown in broth culture and in tissue culture cell lines. PtK_2 cells (*Potorous tridactylis*; ATCC CCL 56) were cultured in minimum essential medium (GIBCO) supplemented with 10% fetal calf serum, glutamine, and nonessential amino acids in the absence of antibiotics. For infection experiments, cells were grown on 12-mm coverslips in six-well tissue culture dishes and placed in serum-free medium 2 h prior to infection. For the infection, 5 μl of an exponentially growing bacterial culture (optical density at 600 nm = 0.6) was added directly to the culture dishes. One hour later, the coverslips were rinsed thrice with prewarmed complete minimum essential medium supplemented with 25 μg of gentamicin per ml and incubated for 2 to 4 h in the same medium. The coverslips were then rinsed in PBS, and the cells were fixed in 3.7% formaldehyde in PBS and permeabilized with 0.2% (vol/vol) Triton X-100 in PBS. Coverslips were processed for double fluorescence microscopy by using fluorescein-labeled phalloidin and the affinity-purified polyclonal ActA antibodies followed by rhodamine-labeled goat anti-rabbit antibodies (Dianova).

For the preabsorption of the ActA antiserum, 10 confluent PtK_2 tissue culture dishes (diameter, 100 mm) were fixed and permeabilized as described above. The antiserum was diluted 10-fold in PBS and incubated consecutively with each semidry culture dish for 30 min. The antiserum was used at a final dilution of approximately 1:500; the affinity-purified antibodies were used at 20 μg/ml.

For staining of live listeriae, exponentially growing cultures were harvested by centrifugation and washed twice with PBS. Coverslips (12 mm) were coated with poly-L-lysine (1 mg/ml) and incubated with 50 μl of the bacterial suspension for 5 min. After the coverslips were rinsed in PBS, the adherent bacteria were incubated for 30 min with the diluted affinity-purified ActA antibodies. The coverslips were then rinsed thrice with PBS, fixed with formaldehyde, rinsed again with PBS, and processed with the secondary antibody as described above. Samples were examined with a Zeiss Axiophot microscope equipped with epifluorescence. Photographs were taken with Ilford 400 Delta films.

Immunoelectron microscopic labeling studies. Mouse macrophages (J774 A.1; ATCC TIB 76) were cultured in the absence of antibiotics in Dulbecco modified Eagle medium (GIBCO) with 10% fetal calf serum, and infection with *L. monocytogenes* EGD was performed as described above. The infected macrophages were fixed with formaldehyde and glutaraldehyde directly in petri dishes and embedded by progressive lowering of temperature as described earlier (3).

Ultrathin sections were mounted onto Formvar-coated nickel grids (300 mesh), incubated with affinity-purified polyclonal ActA antibodies (200 μg/ml) overnight at 4°C, washed with PBS, and then incubated with protein A-gold complexes (gold particle size, 10 nm; at an A_{520} of 0.02) for 1 h at room temperature. Subsequently, the grids were rinsed with PBS containing 0.01% Tween 20 and then with distilled water and air dried. Counterstaining was performed with 4% aqueous uranyl acetate for 5 min. The samples were examined with a Zeiss electron microscope 10 B at an acceleration voltage of 80 kV at calibrated magnifications.

RESULTS

Characterization of antisera against synthetic ActA peptides and attempts to purify ActA from culture supernatants. Previously we have raised antisera against synthetic peptides corresponding to amino acid residues 41 to 54 and 91 to 103 of the ActA polypeptide. This enabled us to identify both the ActA protein and a truncated derivative in trichloroacetic acid-precipitated culture supernatants of the *L. monocytogenes* EGD strain and the isogenic *actA1* mutant, respectively (3). Repeated boosting of immunized rabbits with ovalbumin-conjugated peptides led to the production of antisera that showed specific reaction with the 90-kDa ActA

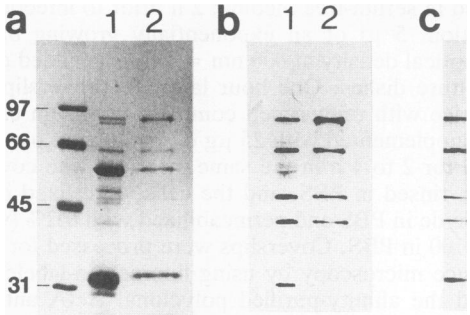


FIG. 1. Immunoblot analysis of ActA polypeptides enriched by ammonium sulfate precipitation. (a) Coomassie blue-stained SDS–10% polyacrylamide gel with molecular weight standards (left lane), concentrated culture supernatant of *L. monocytogenes* (lane 1), and the precipitate of the supernatant obtained with 40% ammonium sulfate saturation (lane 2). (b) Corresponding immunoblot reacted with anti-ActA peptide (amino acids 91 to 103) antiserum. (c) Corresponding immunoblot of concentrated culture supernatant reacted with MAb N111.

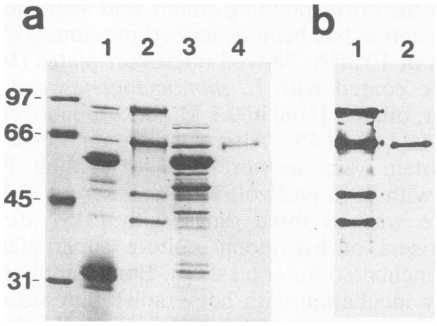


FIG. 2. Purification of ActA from concentrated supernatants of *L. monocytogenes* EGD and of the truncated ActA1 polypeptide of the isogenic *actA1* mutant by immunoaffinity chromatography. (a) Coomassie blue-stained SDS–10% polyacrylamide gel with *L. monocytogenes* EGD supernatant (lane 1), ActA polypeptides eluted with 3.7 M $MgCl_2$ from immobilized MAb N81 after incubation with EGD supernatant (lane 2), *L. monocytogenes actA1* supernatant (lane 3), and ActA1 polypeptides eluted from immobilized MAb N81 with 3.7 M $MgCl_2$ after incubation with *actA1* supernatant (lane 4). Molecular weight standards are shown in the left lane. (b) Corresponding immunoblots of the eluted ActA polypeptides (lane 1, corresponding to lane 2 in panel a) and ActA1 polypeptides (lane 2, corresponding to lane 4 in panel a) reacted with MAb N111.

polypeptide and four other major cross-reacting bands, with apparent molecular sizes of 63, 48, 41, and 33 kDa, in culture supernatants concentrated more than 100-fold by ultrafiltration (Fig. 1). The specific cross-reactivity of these peptide antisera with ActA polypeptides was used to screen various ammonium sulfate fractions obtained from concentrated supernatants for the presence of the ActA polypeptides. The ActA protein (90 kDa) and three of its major degradation products (63, 48, and 41 kDa) (see below) were found by immunoblotting to be highly enriched in the 40% ammonium sulfate precipitant (Fig. 1a, lane 2). The 33-kDa polypeptide, cross-reacting with the anti-ActA peptide antiserum, was not detected in this fraction and was found to precipitate with most other *L. monocytogenes* supernatant proteins at 60% ammonium sulfate saturation (data not shown).

Despite the strong enrichment of ActA, subsequent purification of the 90-kDa ActA polypeptide was hampered by the presence of the apparent degradation products of ActA and by erratic behavior of this polypeptide on the various chromatographic columns employed.

Purification of ActA by immunoaffinity chromatography and production of polyclonal antibodies. These difficulties prompted us to consider immunoaffinity chromatography using specific MAbs as an alternative approach to purify the ActA polypeptides. To do this, mice were first immunized with the 90-kDa ActA polypeptide isolated by electroelution from preparative SDS-PAGE gels. Hybridoma supernatants were screened by ELISA using enriched ActA fractions, obtained by ammonium sulfate precipitation, as coating antigens. Positive hybridoma supernatants were further analyzed by immunoblotting using concentrated crude bacterial supernatants. Three independently isolated clones (designated N20, N81, and N111) were selected for further characterization. By isotype analysis, these MAbs were identified as immunoglobulin G1 carrying the kappa light chain. As observed with the ActA-specific antipeptide antiserum, the three MAbs isolated in this study also reacted with the 90-kDa protein and the distinct "ladder" of degradation products (Fig. 1c). When these MAbs were tested on PtK_2 cells infected with *L. monocytogenes* and processed for immunofluorescence microscopy after formaldehyde fixation (see Materials and Methods), none of the antibodies was capable of labeling intracellular bacteria.

Since MAbs often fail to react with aldehyde-fixed material, a prerequisite for electron microscopic studies, we next attempted to purify the ActA polypeptide by immunoaffinity chromatography with the available MAbs, with the aim of raising suitable ActA-specific polyclonal antisera. To select for the appropriate ActA MAb, the purified MAbs N20, N81, and N111 were immobilized and incubated with the concentrated supernatants fluids of *L. monocytogenes* EGD. Only immobilized MAb N81 was found to recognize and bind the native ActA polypeptides, permitting enrichment on the Sepharose matrix (data not shown). Following overnight incubation of concentrated bacterial supernatant fluids with immobilized N81 MAb, the affinity matrix was processed as described in Materials and Methods. Analysis by SDS-PAGE demonstrated that the 33-kDa ActA fragment was eluted entirely within early fractions, in contrast to the higher-molecular-mass ActA fragments and the mature ActA polypeptide. These cross-reacting bands corresponded to major polypeptides in this fraction, as detected by Coomassie blue staining of the SDS-PAGE gel (data not shown). Repeated centrifugation in Centricon tubes allowed the removal of $MgCl_2$ and further concentration of pooled fractions (Fig. 2a, lane 2). By using the same procedure, it was also possible to purify the truncated ActA1 polypeptide from the culture supernatants of the *actA1* mutant strain (Fig. 2a, lane 4).

At this point it was important to establish if these copurifying polypeptides were indeed bona fide degradation products of the ActA protein. Hence, a fraction containing these polypeptides was electrophoresed and blotted onto polyvinylidene difluoride membranes, and the N-terminal amino acid sequences were determined. The sequence of the first 20 amino acids obtained for all polypeptides matched the predicted amino acid sequence of mature ActA starting at amino acid 30. Hence, the 63-, 48-, and 41-kDa polypeptides must have been generated from the mature 90-kDa polypeptide by C-terminal processing.

Localization studies. The purified ActA polypeptides were

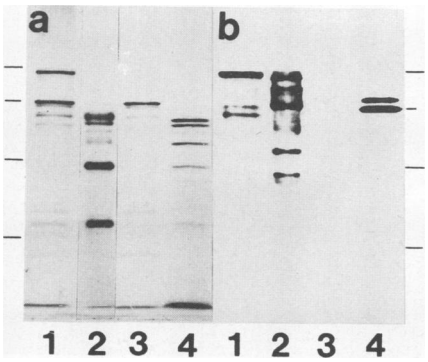


FIG. 3. Detection of ActA associated with the cell wall (lanes 1 and 3) and in trichloroacetic acid-precipitated supernatants (lanes 2 and 4) of *L. monocytogenes* strain EGD (lanes 1 and 2) or its isogenic *actA1* mutant (lanes 3 and 4). Bacterial pellets were harvested and placed directly into boiling SDS-sample buffer. (a) Coomassie blue-stained SDS–10% polyacrylamide gel; (b) corresponding immunoblot reacted with the polyclonal anti-ActA antiserum and developed by using a chemiluminescent substrate. Note that little degradation of the cell wall-associated ActA has occurred (lane 1). Molecular weight markers are indicated (top to bottom: 97, 66, 45, and 31 kDa).

then used to obtain a specific rabbit polyclonal anti-ActA antiserum as described in Materials and Methods. We first examined the localization of the ActA protein on *L. monocytogenes* EGD during exponential growth in brain heart infusion cultures at 37°C, i.e., under conditions in which optimal expression of ActA occurred. Immunoblot analysis using the ActA-specific polyclonal antibodies showed that the ActA polypeptide was present both in SDS cell wall extracts of whole cells and in the supernatant fluids of these bacteria (Fig. 3, lanes 1 and 2). To a large extent the ActA polypeptide was present in the cell wall extracts as judged by Coomassie blue staining of the corresponding SDS-PAGE gel. This could be expected on the basis of primary sequence prediction of a membrane-based anchor at its C-terminal end. These results were corroborated by the finding that the mutant ActA1 polypeptide lacking 214 amino acids at its C-terminal end was secreted entirely into the supernatant fluids of the growing mutant bacteria (Fig. 3, lanes 3 and 4). In contrast to the ActA present in the supernatant fluids, little degradation of cell wall-associated ActA was observed when bacterial pellets were directly placed into boiling SDS-sample buffer (cf. lanes 1 and 2 in Fig. 3).

We next examined the ability of the antiserum to recognize native ActA on the surface of living bacterial cells. Exponentially growing cells of the wild-type strain or its isogenic *actA1* mutant, grown as described above, were adsorbed onto poly-L-lysine-coated coverslips and then incubated with anti-ActA antiserum; this was followed by formaldehyde fixation. Labeling of the bacteria was detected by using rhodamine-conjugated secondary antibodies. As can be seen in Fig. 4 (g through j), the anti-ActA polyclonal antiserum was clearly capable of recognizing native ActA on the surface of growing bacteria. As expected, the secreted ActA1 polypeptide was not detected on the surface of mutant bacteria.

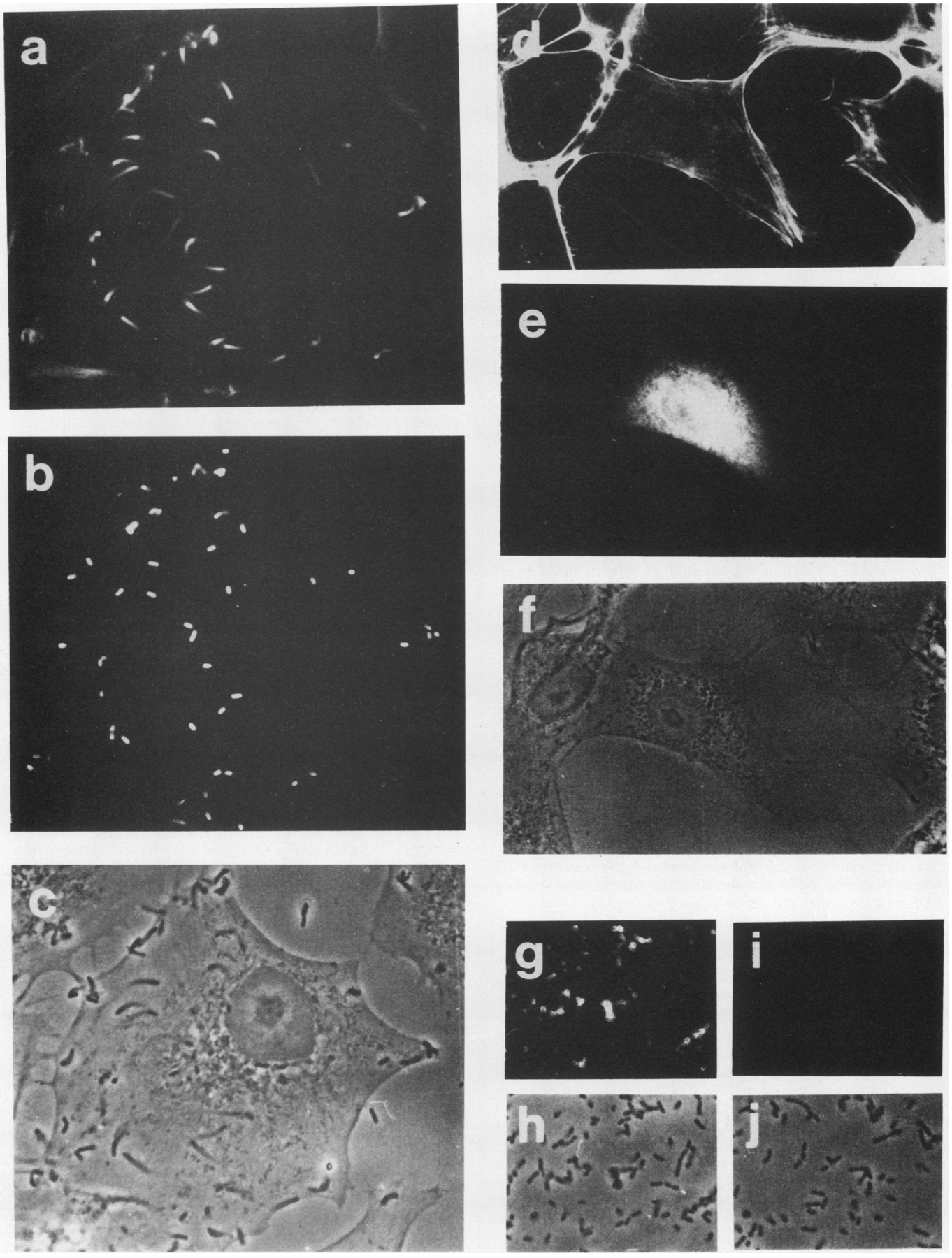
These results encouraged us to examine the localization and distribution of the ActA polypeptide in PtK_2 epithelial cell lines infected with either wild-type bacteria or the corresponding *actA1* mutant. Because of nonspecific cross-reactions of crude antisera, the ActA antiserum was extensively preabsorbed on noninfected PtK_2 cells to prevent spurious staining of the host cell components. Alternatively, affinity-purified polyclonal ActA antibodies were applied. In cells infected with the wild-type strain, phalloidin staining of bacterial actin tails always corresponded with the staining of infecting bacteria by the anti-ActA antiserum at the leading edge of the actin tail (Fig. 4a through c). Surprisingly, no ActA could be detected within these actin tails. The ActA always seemed to be tightly associated with the bacterial surface regardless of the time spent by individual bacteria in the infected host cell. The preabsorbed ActA antiserum and the affinity-purified ActA antibodies revealed identical staining patterns. In host cells infected with the *actA1* mutant, where the infecting bacteria grew as microcolonies, diffuse staining of the host cytoplasm was observed, indicating production and secretion of the C-terminally truncated ActA1 polypeptide from these bacteria into the host cytoplasm (Fig. 4d through f).

Immunoelectron microscopic studies on infected mouse J774 macrophages, using protein A-gold complexes and affinity-purified polyclonal ActA antibodies, indicated the presence of the ActA polypeptide on the bacterial surface at all stages of the infection cycle (Fig. 5a through d). Even bacteria within the phagolysosomal compartment prior to escape into the host cytoplasm were found to harbor the ActA polypeptide on their surface. Bacteria which exhibited actin accumulation around the cells showed random distribution of ActA on the bacterial cell surface; no ActA polypeptide was detected by the methods applied in the surrounding actin sheath or in the actin tail (Fig. 5e through j). Hence, the ActA polypeptide is probably required in the initiation of actin accumulation by the bacterium following the infection of the host cell.

Detection of the ActA polypeptide in *L. monocytogenes* strains of different serotypes. The presence of the ActA polypeptide in *L. monocytogenes* has to date been shown to be present in two serovars, namely, 1/2a and 1/2c (3, 7). It was therefore of interest to determine its presence and localization in strains of various *L. monocytogenes* serotypes. To do this, SDS cell wall extracts from exponentially growing cells were loaded onto SDS-PAGE gels, immunoblotted, and reacted with the anti-ActA rabbit antiserum. Immunostaining with peroxidase-labeled secondary antibodies showed that all *L. monocytogenes* strains except a serotype 3a strain and a serotype 4ab strain expressed the ActA polypeptide (Fig. 6; Table 1). There were small but clear differences in the molecular weights of the various ActA polypeptides, and each strain showed a distinct pattern of reactivity of ActA-derived degradation products. Further analysis of the serotype 3a and 4ab strains showed that the ActA polypeptide was also absent in their respective culture supernatants (data not shown).

DISCUSSION

The ActA polypeptide is a major virulence factor of *L. monocytogenes* that is required for actin accumulation on the surface of intracytoplasmic bacteria as a prerequisite for intracellular movement and cell-to-cell spread. In this communication, we described a simple method to purify the ActA polypeptide from culture supernatants in a single step by using immunoaffinity chromatography performed with a MAb isolated in this study. By using specific anti-ActA polyclonal antisera, it was found that ActA was present on the surface of the bacteria irrespective of whether they were growing in broth culture or in the infected culture cell lines.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The ActA polypeptide was not detected in the actin tails of intracellularly moving *Listeria* bacteria. Immunoblotting experiments showed that the ActA polypeptide was present on the surfaces of various serovars of *L. monocytogenes* and led to the detection of a naturally occurring variant strain that did not produce this important bacterial virulence factor.

The presence of multiple ActA-derived breakdown products and the erratic behavior of ActA on various chromatographic substrates forced us to examine alternative means of purifying the ActA polypeptide. Hence, the 90-kDa ActA polypeptide was first electroeluted from preparative SDS gels, and this fraction was used to produce anti-ActA MAbs. Subsequently, the abilities of these MAbs to serve as affinity substrates to purify the ActA polypeptide were examined. One of the MAbs, N81, did indeed prove to be suitable for the immunochromatographic purification of ActA and its C-terminally truncated ActA1 polypeptide from supernatant fluids of the respective bacteria. This method also resulted in the copurification of the 63-, 48-, and 41-kDa polypeptides, all of which were shown by N-terminal end sequencing to be derived from the 90-kDa ActA polypeptide by C-terminal-end-processing events, suggesting that a cosecreted listerial protease may be responsible for this degradation. The Mpl metalloprotease, the only known secreted listerial protease to date, was previously shown to be required for the processing of the pro-PlcB phospholipase to give the mature active PlcB product (13, 16) and is hence the prime candidate for the proteolytic processing of the ActA polypeptide observed here. A definitive role for this protease in degrading the ActA polypeptide and its physiological consequences can be obtained only by the analysis of an isogenic *mpl* mutant of *L. monocytogenes* EGD.

Since none of the MAbs produced in this study proved capable of labeling bacteria growing in infected cultured cells under the applied fixation conditions, specific polyclonal antisera against the affinity-purified ActA polypeptides were raised. By using SDS extraction conditions that selectively remove cell surface proteins (7), it was found that the majority of the ActA polypeptide is present on the surface of the growing bacteria, with small amounts present in the supernatant fluids. (Although these cell wall extracts appear to be an ideal starting point for isolation of the ActA polypeptide, SDS is difficult to remove from such extracts, rendering them inappropriate for further functional characterization.) In contrast, the isogenic *actA1* mutant produces a C-terminally truncated ActA polypeptide which was found only in the supernatant fluids of the growing mutant bacteria. These observations are in agreement with predictions from the primary structure of the ActA polypeptide that indicated an alpha-helical hydrophobic anchor located close to its C-terminal end. These biochemical results were confirmed by light microscopy-based immunofluorescence studies that showed that the ActA polypeptide was exposed on the surface of cells growing in broth culture.

Extensive purification of the anti-ActA antiserum by preabsorption on noninfected PtK_2 epithelial cells also permitted detection and localization of the ActA polypeptide on bacteria within infected PtK_2 and J774 cells. Regardless of the stage within the infection cycle of the infecting bacterium, staining for the ActA polypeptide was always associated with its even distribution on the surface of the bacteria. There was no evidence of polarized distribution of the ActA protein on the bacterial surface, nor was it found to be associated with the bacterial actin tail. Although it could be argued that the ActA within the actin tail is modified, degraded, or complexed with a host cell protein and thus not accessible to the antibody, several observations suggest that this may not be the case. First, the antiserum was produced against and recognizes all major breakdown products of the ActA polypeptide (Fig. 3). Second, the truncated ActA1 polypeptide produced by the isogenic mutant is clearly detected by this antiserum when it is secreted into the surrounding host cytoplasm. Third, immunoelectron microscopic studies on cross-sections of the infected J774 cells with the anti-ActA antiserum showed labeling only of the surface of the infecting *L. monocytogenes* bacteria. Taken together, these results suggest that the ActA polypeptide is required at a very early stage of actin accumulation by the bacterium. We suggest that regions of the ActA polypeptide exposed on the bacterial surface may be sites for the concentration of interacting factors that in fact induce actin accumulation and polymerization to generate the subsequent polarization of the actin filaments. The isolation of an *L. monocytogenes* mutant that is able to polymerize actin but unable to generate the actin tails is in keeping with the notion that additional bacterial products are required for intracellular movement (9).

Examination of several strains of *L. monocytogenes* of different serotypes showed that the ActA polypeptide was also present on the surfaces of these bacteria. Nevertheless, microheterogeneity in the molecular weights of the ActA polypeptides of the different strains was observed, and each strain had in turn a distinct pattern of ActA degradation products as detected by the anti-ActA antiserum. Whether these differences in molecular weights are associated with an increase in number of proline repeats or merely insertions and/or deletions within various regions of the ActA molecule or whether they lead to differences in functional efficiency of ActA and hence virulence of a particular isolate is a matter of speculation and will be addressed in further studies. Another aspect that deserves investigation is whether the specific degradation patterns observed for this important virulence factor are clonal with respect to infecting bacterial populations and can be used as a marker in epidemiological studies.

FIG. 4. (a through f) Localization of ActA in PtK_2 cells infected with *L. monocytogenes* EGD (a through c; magnification, ×1,200) or its isogenic *actA1* mutant (d through f; magnification, ×600). Four hours after the start of infection, cells were processed for double immunofluorescence microscopy by using fluorescein isothiocyanate-labeled phalloidin (a and d) and the rabbit polyclonal anti-ActA antiserum followed by rhodamine-labeled secondary antibodies (b and e). Panels c and f show the corresponding phase-contrast micrographs. Within the infected cells, ActA is detected only around the bacteria (b) and not in the bacterium-induced comet tails (compare panels a and b), whereas the truncated ActA1 polypeptide is diffusely distributed throughout the cytoplasm of the host cell. A number of bacterial cells adhering to the plastic substratum are not stained by the anti-ActA antiserum (compare panels b and c). These are adherent bacteria that have been killed by the bactericidal effect of gentamicin in the culture medium. (g through j) Localization of ActA on the surface of living listeriae. *L. monocytogenes* EGD (g and h) or its isogenic mutant (i and j) were adsorbed onto poly-L-lysine-coated coverslips and incubated with the affinity-purified polyclonal antibodies. After formaldehyde fixation, bound primary antibodies were detected with rhodamine-labeled secondary antibodies (g and i). Panels h and j show the corresponding phase-contrast micrographs (magnification, ×1,000).

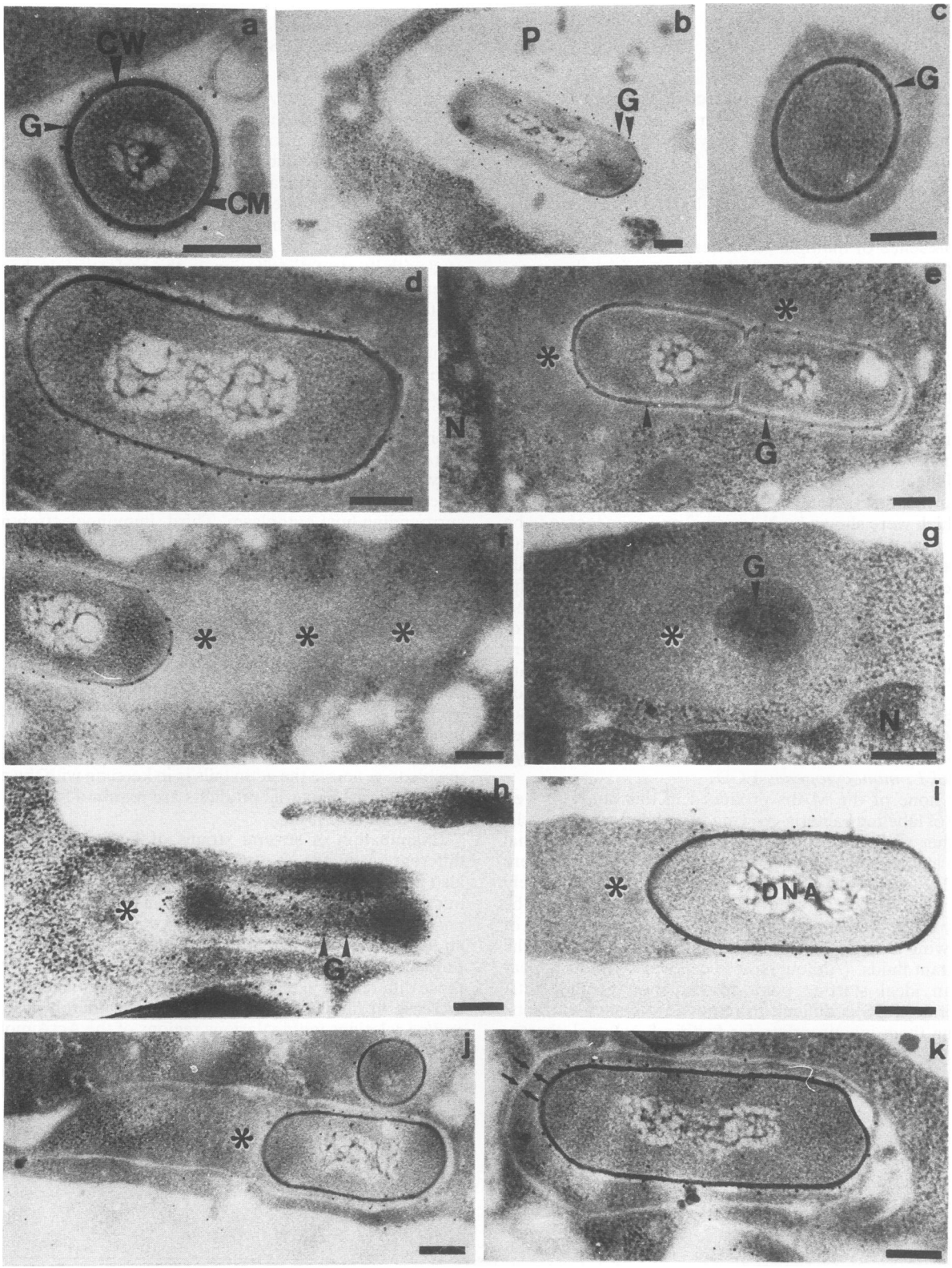

FIG. 5. Immunoelectron microscopic detection of the ActA polypeptide in infected J774 cells by using affinity-purified polyclonal ActA antibodies and protein A-gold complexes (10 nm). This figure depicts the ActA polypeptide localization on the surface of bacterial cells in an entire infection cycle of *L. monocytogenes* EGD. (a) Bacterial cells attached to macrophages show ActA distributed on the cell surface. (b) *L. monocytogenes* expresses the ActA polypeptide on its surface even in the phagolysosomal compartment. (c and d) Cross-sections (c) and longtitudinal sections (d) of freely residing bacteria in the cytoplasm of the macrophage also exhibit random distribution of the ActA polypeptide on their surface. (e) Replicating bacterial cells express ActA randomly on their surface; note that actin accumulation (*) at one pole of the bacterium does not alter the ActA distribution pattern. (f) A bacterium with an actin tail. Again, no ActA is detectable in the tail; note the random distribution of ActA on the bacterial surface. (g and h) Cross-sections (g) and longitudinal sections (h) of the bacterial cell surface, showing the ActA distribution only on the surface of the bacterium. (i) A protrusion with a bacterium at its tip and an actin tail; note the lack of ActA labeling in the actin tail. (j) Protrusion penetrating an adjacent macrophage (intercellular spread). (k) Protrusion engulfed in a double membrane consisting of the cytoplasmic and protrusion membranes (arrows). Abbreviations: CW, cell wall; CM, cytoplasmic membrane; G, gold particle; N, nucleus; P, phagolysosome. Bars, 200 nm.

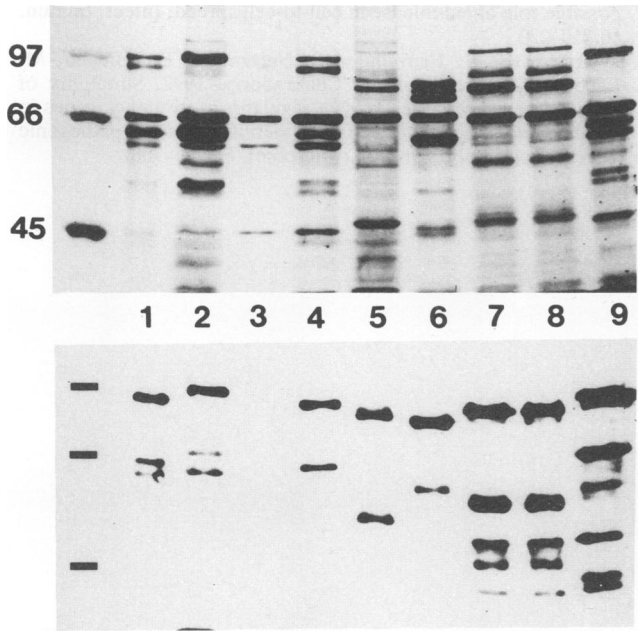

FIG. 6. Detection of ActA in SDS cell wall extracts of *L. monocytogenes* strains of various serotypes. Bacterial pellets were extracted with 1% SDS in PBS for 15 min at 37°C. (Top) Silver-stained SDS–7.5% polyacrylamide gel, with molecular weight markers shown on the left lane. (Bottom) Corresponding immunoblot reacted with the ActA antiserum and developed with a chemiluminescent substrate. The following *L. monocytogenes* strains (see also Table 1) were used: EGD (lanes 1), NCTC 7973 (lanes 2), SLCC 2373 (lanes 3), SLCC 2479 (lanes 4), SLCC 2374 (lanes 5), SLCC 1444 (lanes 6), SLCC 2376 (lanes 7), SLCC 4925 (lanes 8), and SLCC 2482 (lanes 9). Note the identical ActA reaction patterns for the two serotype 4c strains, SLCC 2376 (lanes 7) and SLCC 4925 (lanes 8).

Of the two strains that did not react with the specific anti-ActA antisera, the serotype 4ab strain is nonhemolytic and avirulent, has previously been shown to lack all regions corresponding to the virulence region of *L. monocytogenes*, and is in all likelihood not an *L. monocytogenes* strain (20). The SLCC 2373 serotype 3a strain, on the other hand, is hemolytic, but ActA cannot be detected either in supernatant fluids or in the cell wall of these bacteria. This strain has previously been characterized as a hemolytic but nonvirulent strain of *L. monocytogenes* (6). We are currently examining the genetic basis of the lack of expression of ActA by this strain.

In conclusion, we have reported a simple and rapid method of purifying the ActA polypeptide by immunoaffinity chromatography. This method should allow the isolation of various truncated and mutant forms of the ActA polypeptide that will be required for the analysis of its biological activity. Our current studies are directed to the use of immunoprecipitation techniques with the antibodies produced in this study to examine the expression of ActA at various times following infection of the eucaryotic host cell. Finally, the results of ActA localization within the eucaryotic cell shown herein are the first to be reported for a virulence factor expressed by an intracellular bacterial pathogen and will help us in understanding similar processes in other intracytoplasmic bacteria.

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